

Comparisons of Selected Stains for Distinguishing Between Live and Dead Eggs of the Plant-parasitic Nematode *Heterodera glycines*¹

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ABSTRACT: Seven stains were tested on *Heterodera glycines* (soybean cyst nematode) to determine whether live and dead eggs of a plant-parasitic nematode could be distinguished from each other. When observed with bright-field microscopy, the stains chrysoidin, eosin-Y, new blue R, and Nile blue A were useful in differentiating dead from live eggs, particularly when the stains were combined with dimethyl sulfoxide. Acridine orange, eosin-Y, fluorescein, and fluorescein diacetate, when observed with fluorescence optics, also differentially stained live and dead eggs.

KEY WORDS: *Heterodera glycines*, soybean cyst nematode, nematode egg stains, nematode egg viability, fungus-nematode interactions, microscopy.

Evaluation of the pathogenicity and virulence of biological control agents of plant-parasitic nematodes often requires that live and dead nematodes be distinguished from each other. When nematode eggs, rather than motile vermiform nematodes, are studied, it can be particularly difficult to determine which individuals are alive and which are not. A number of stains have been used to help establish whether a nematode is dead or alive; however, as summarized by Hooper (1986), varying results have been reported for some of these stains. To test for fungal pathogenicity on eggs of plant-parasitic nematodes, it is therefore necessary to determine which stains are the most reliable for differentiating dead and live eggs of these nematodes. To this end, we applied different stains to eggs of the soybean cyst nematode, *Heterodera glycines*.

Seven stains were tested on live eggs and on eggs that had been heated-killed or that had died of natural causes. Six of these stains had been used on plant-parasitic or free-living nematodes in earlier studies. One stain—aqueous fluorescein—has not, to our knowledge, been previously reported as a stain for plant-parasitic nematodes. Even though some of these stains have been reported to be ineffective or inconsistent under certain circumstances, we evaluated them to determine whether they could be useful for studies of this nature. Because difficulty with infiltration might be one cause for variable results that have been reported in the literature, 6 of the stains

were applied both with and without dimethyl sulfoxide (DMSO) to determine whether this chemical would aid penetration of stains into eggs. The objectives of this study were to find a stain that would (1) penetrate live or dead plant-parasitic nematode eggs and give a clear distinction between them, (2) be effective within an hour or 2 after application, and (3) stain fungal hyphae.

Materials and Methods

NEMATODE CULTURE AND PREPARATION OF EGGS FOR STAINING: Eggs of *Heterodera glycines* Ichinohe, races 3 and 5, were obtained from previously established monoxenic root explant cultures (Lauritis et al., 1982). Nematodes were maintained in vitro on excised root tips of *Glycine max* (L.) Merr. cv. Kent grown on Gamborg's B-5 medium (Gamborg et al., 1976; Huettel and Rebois, 1985). Egg-containing cysts from these cultures were placed into 250- μ l tubes and disrupted in distilled water with a hand-held homogenizer. The resulting egg suspensions were then either removed from the tubes and stained without further treatment, or left in the tubes, immersed in boiling water for at least 5 min to heat-kill the eggs, and then removed and stained.

STAINS: The stains prepared were 200 ppm acridine orange (Homeyer, 1953), 0.005% chrysoidin (Doliwa, 1956, as cited in Hooper, 1986), 0.67% eosin-Y (Chaudhuri et al., 1966), 0.01% fluorescein diacetate in phosphate buffer (pH 7.3) (Bird, 1979), 0.05% new blue R (Shepherd, 1962), 0.1% Nile blue A (Ogiga and Estey, 1974), and 0.01% fluorescein. All were in aqueous solution unless otherwise stated. Acridine orange, chrysoidin, eosin-Y, and Nile blue A were also prepared in 10% aqueous DMSO. Fluorescein diacetate was made in 10% DMSO in 0.067 M phosphate buffer, and new blue R was prepared as 0.045% in 10% aqueous DMSO. DMSO was not added to fluorescein.

STAIN TECHNIQUE: For each stain test, approximately 20 μ l of egg suspension were placed on a slide and mixed with a drop of stain. Unstained eggs were mixed with a drop of distilled water. Staining was al-

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lowed to proceed for at least 2 hr, and the eggs were then examined with either a Nikon Microphot FX for bright-field microscopy or a Nikon Diaphot-TMD for fluorescence microscopy. Bright-field stains without DMSO were each tested on a minimum of 4 slides of egg suspensions. Each bright-field stain with DMSO was tested on at least 6 slides. Acridine orange was an exception—it was tested 4 times with DMSO. Fluorescent stains without DMSO were each tested a minimum of 3 times; with DMSO, each fluorescent stain was tested on at least 2 slides of egg suspensions.

Fluorescence was observed using epifluorescence optics and 5 different filter systems. The filter systems are referred to in this paper as B, G, V, UV, and BV. The composition of these filter systems was as follows: B—blue light excitation (dichroic mirror 510, excitation filter 420–490, barrier filter 520), G—green excitation (dichroic mirror 580, excitation filter 546/10, barrier filter 580), V—violet excitation (dichroic mirror 430, excitation filter 380–425, barrier filter 460), UV—ultraviolet excitation (dichroic mirror 400, excitation filter 330–380, barrier filter 420), and BV—blue violet excitation (dichroic mirror 455, excitation filter 400–440, barrier filter 480). The B, G, V, UV, and BV filter combinations were all used for each of the fluorescent stains in this study. Fluorescence was observed with objective lenses of 20 \times and 40 \times magnification. Most of the dead eggs examined with the fluorescence microscope were heat-killed, and the colors reported in Table 1 are based mainly on those eggs. Observations of fungal-infected eggs are included in the text of this paper.

PREPARATION OF FUNGAL-INFECTED EGGS: Dr. L. R. Krusberg (University of Maryland) graciously provided field soil containing cysts of the soybean cyst nematode. Fungi were isolated from cysts and eggs and maintained in pure culture. For bright-field microscopy, yellow and brown egg-containing cysts from cultures over 2 mo old were placed on water agar in petri dishes and inoculated with 2 unidentified fungi. In the first experiment, the inoculated cysts were placed into a 25°C incubator for 3.5 wk. The cysts inoculated with the second fungus were incubated for 6 wk. For fluorescence microscopy, egg-containing cysts were inoculated with a third fungus and placed into the incubator for 2 wk. Following incubation in all of these experiments, the inoculated cysts were disrupted and the eggs were stained.

Results

BRIGHT-FIELD MICROSCOPY: When observed with bright-field microscopy, shells of both hatched and unhatched eggs tended to stain a little darker than the background stain color. In many of the live eggs, the shell stained but the larva inside the egg was unstained. Figure 1 is an example of this type of staining pattern in a live egg. The major color differences described here between live and dead eggs were observed in the egg contents. In eosin-Y, shells were pink, live eggs (Fig. 1) clear to pink, and dead eggs (Fig. 2) pink to dark pink. In acridine orange, shells

appeared yellow, live eggs clear to yellow, and dead eggs dark yellow, orange, yellow and orange mixed, or orange-yellow. With chrysoidin, shells stained yellow, live eggs clear to yellow, and dead eggs (Fig. 3) dark yellow (often with orange droplets), or orange. Nile blue A stained shells blue, live eggs (Fig. 4) clear to blue, and dead eggs (Fig. 5) dark blue, turquoise, or mixed dark blue and orange. Shells in new blue R were blue or purple, live eggs were clear, blue, or purple, and dead eggs (Figs. 6, 7) were dark blue, dark purple, dark bluish-purple, or blue and orange.

Live eggs that had picked up some stain and dead eggs that were not darkly stained were sometimes difficult to distinguish from each other. When DMSO was used, the colors observed appeared similar to those seen when the stains were not mixed with DMSO. However, the distinction between live and dead eggs became clearer, because the dead eggs generally appeared much darker than the live stained eggs. This was most noticeable when DMSO was added to chrysoidin, eosin-Y, new blue R, and Nile blue A. To determine whether 1 or more of these 4 stains was superior to the others for fungal biological control studies, DMSO was added to each stain and the stains were applied to eggs inoculated with 2 different fungi. Some of these eggs had presumably died of natural causes, including fungal-induced death. A total of 416 eggs was counted for each stain—208 eggs for each inoculation experiment. When the results of the 2 inoculation experiments were combined for each stain, the percentages of live eggs were as follows: chrysoidin + DMSO—91.1%, eosin-Y + DMSO—92.3%, new blue R + DMSO—92.5%, Nile blue A + DMSO—92.3%.

The fungal hyphae that picked up the stains could be seen the most clearly with new blue R (Fig. 7) and Nile blue A. When chrysoidin and eosin-Y were used, the stained hyphae tended to be similar in color to the background color on the slides.

FLUORESCENCE MICROSCOPY: The colors observed with fluorescence microscopy are indicated in Table 1, and examples of eggs treated with stains are shown in Figures 8–19. The fluorescence colors often appeared brighter when observed with the 20 \times objective lens than when observed with the 40 \times lens.

Live and dead eggs that had not been treated with a stain were difficult to distinguish from each other. In some filter systems (particularly the G), the fluorescence was either faint or faded

fairly quickly. The dead eggs were often brighter than the live eggs, especially when the B and BV filter systems were used. However, live eggs in groups also tended to be brighter than single live eggs, and therefore sometimes hard to distinguish from dead eggs.

Acridine orange, eosin-Y, fluorescein, and fluorescein diacetate all differentially stained live and dead nematode eggs. When DMSO was present, fluorescence colors or patterns did not always appear the same as those observed without DMSO. For example, dead eggs treated with acridine orange and DMSO and observed with the V filter system were greenish-yellow, orange, red, or red-orange. However, the differences that resulted from the addition of DMSO did not appear to enhance the usefulness of the stains.

Acridine orange combined with the BV filter system (Figs. 9, 10) appeared to give clearer distinctions between live and dead eggs than when used with the other filter systems. However, the other filter systems did give some distinctions with acridine orange. Overall, this was not the most effective stain used in this study. Eosin-Y distinguished between live and dead eggs with all of the filter systems, as the live eggs did not fluoresce. However, when the B and UV filter systems were used, not all of the dead eggs fluoresced, either. Consequently, the G, V (Fig. 8), and BV filter systems appeared to be the most reliable with the eosin-Y stain.

When fluorescein was used as the stain, the live and dead eggs were most easily distinguished from each other with the V and UV filter systems (Figs. 11, 14, 15). Fluorescein diacetate gave good distinctions between live and dead eggs. The results were clearest with the B and BV filter systems (Figs. 12, 13, 16–19), and with the V system. The G system did not give as distinct a difference between the eggs. With the UV system,

live and dead eggs were generally distinguishable from each other. However, during one of the fluorescence experiments, the fluorescence colors of the live and dead eggs were difficult to tell apart.

Hatched eggs fluoresced in acridine orange. In eosin-Y, fluorescein, and fluorescein diacetate, these shells commonly varied from fluorescent (Fig. 11) to nonfluorescent (Fig. 13), even within a single stain-filter system treatment. In a number of treatments, eggs were observed that did not exhibit a general diffuse fluorescence, or did not fluoresce at all, but that did stand out as dark objects against the bright backgrounds. Examples of such eggs are in Figures 11 and 17 (live eggs) and Figure 12 (hatched egg). Some of these eggs had a faint fluorescence around the perimeter, whereas others had portions of the internal contents that fluoresced (Fig. 18). All of these eggs, whether or not they had any fluorescence, could be more easily discerned than the nonfluorescing eggs represented by the hatched egg in Figure 13.

The fluorescence microscopy results with fungal hyphae were as follows: When stained with acridine orange, the fungal hyphae fluoresced orange with all but the G filter system. When the G system was used, the hyphae fluoresced red. Although the hyphae often fluoresced the same color as the eggs they infected, the hyphae inside the eggs were frequently visible because of the fluorescence pattern they formed. However, internal hyphae (i.e., those inside of eggs) were obscured by the fluorescence of some eggs. Occasionally, hyphae were seen that did not appear to be fluorescing.

With eosin-Y, the hyphae were nonfluorescent to dark against the background with all but the BV filter system. When the dark hyphae filled a dead egg, it was sometimes difficult to see the

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Figures 1–19. Light micrographs of *Heterodera glycines* eggs treated with various stains. Abbreviations: dead egg (D), hatched egg (H), live egg (L). Figures 1–7: bright-field optics. Figures 8–19: fluorescence optics. 1. Live egg treated with eosin-Y. The internal contents of the egg are clear. 2. Dead egg stained with eosin-Y. 3. Dead egg stained with chrysoidin. 4. Live egg stained with Nile blue A. 5. Dead egg stained with Nile blue A. 6. Dead egg stained with new blue R. 7. Dead egg and fungal hyphae (arrows) stained with new blue R. 8. Dead egg stained with eosin-Y. V filter system. 9, 10. Eggs stained with acridine orange. BV filter system. 9. Live egg. 10. Dead egg. 11. Hatched egg and live egg stained with fluorescein. V filter system. Compare with Figures 14 and 15. 12, 13. Hatched egg and dead egg stained with fluorescein diacetate. Compare with Figures 16–19. 12. B filter system. 13. UV filter system. The hatched egg is not fluorescing. The figure demonstrates the difficulty that can arise when total egg populations are being counted, and nonfluorescing eggs are part of the population. 14, 15. Dead egg stained with fluorescein. 14. V filter system. 15. UV filter system. 16–19. Eggs stained with fluorescein diacetate. Figures 17–19 are of the same egg. 16. Dead egg. BV filter system. 17. Live egg. BV filter system. 18. Live egg. B filter system. 19. Live egg. UV filter system.

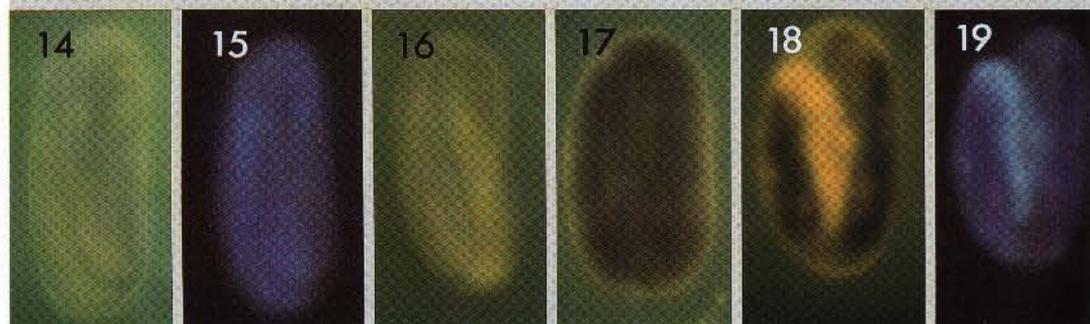
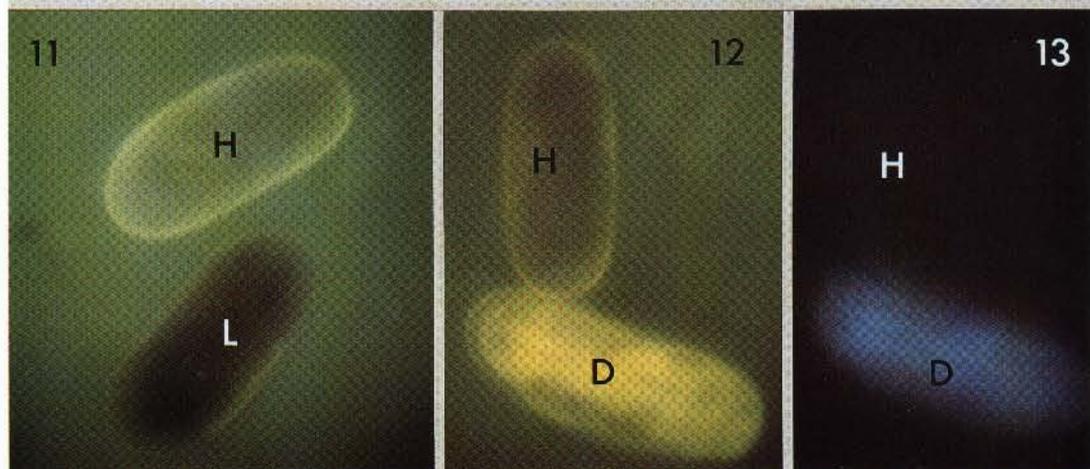
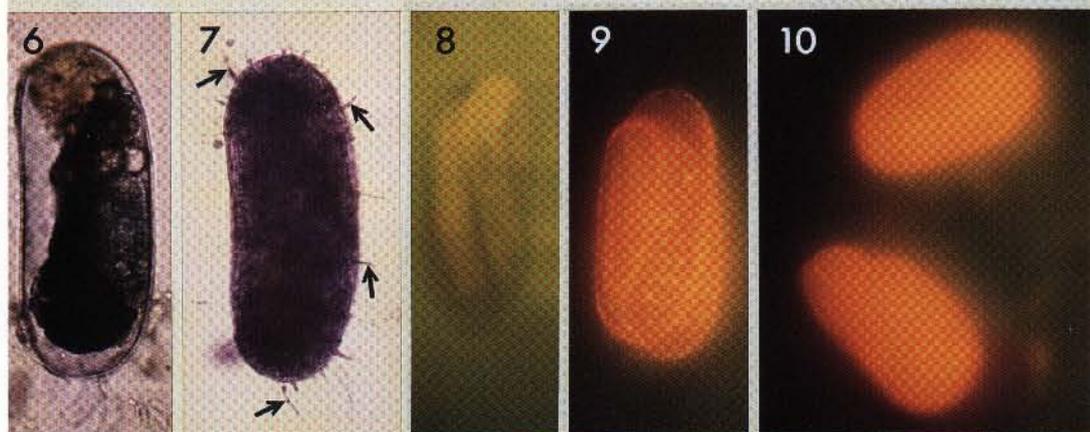
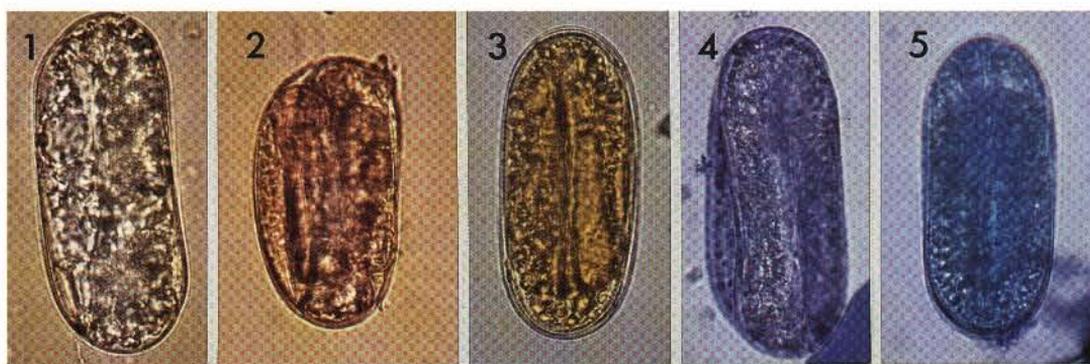


Table 1. Colors observed with the fluorescence microscope in stained and unstained eggs of *Heterodera glycines*.

Stain	Filter system*				
	B	G	V	UV	BV
1. Acridine orange					
Hatched eggs	Orange	Pale red, red	Orange, pale red-orange	Pale red-orange, pale yellow-orange	Orange, red-orange
Live eggs	Orange	Pale red	Orange, red, red-orange	Pale orange, pale orange with green flecks	Orange (some with green or yellow flecks)
Dead eggs†	Orange (some with green or yellow flecks), green, yellow, red-orange	Red	Orange, red with green or yellow flecks, red-orange	Orange, red-orange, yellow-orange	Orange, orange and yellow, red-orange
2. Eosin-Y					
Hatched eggs	Pale red-orange, none	Pale yellow, none	Pale red-orange, none (some visible as dark eggs against bright background)‡	Pale red-orange, none (some dark against background)	None (some dark against background)
Live eggs	None (some dark against background)	None (most dark against background)	None (most dark against background)	None (most dark against background)	None (most dark against background)
Dead eggs	Orange, red-orange, none	Yellow, yellow-orange	Orange with yellow flecks, orange and yellow, red-orange	Orange with yellow flecks, orange and yellow, red-orange, none	Orange (some with yellow flecks), yellow, red-orange
3. Fluorescein					
Hatched eggs	Pale yellow, none (some dark against background)	Pale red-orange, none	Pale yellow, none (some dark against background)	Blue, none	Pale yellow, none (most dark against background)
Live eggs	Greenish-yellow, none (some dark against background)	Pale red-orange, none	None (most dark against background)	Blue to almost none	None (most dark against background)
Dead eggs	Yellow-green, none (dark against background)	Red-orange	Greenish-yellow	Blue, bluish-purple	Greenish-yellow, lime green, some almost none
4. Fluorescein diacetate					
Hatched eggs	Pale greenish-yellow, none (some dark against background)	Pale red, none	Pale yellow, pale orange-yellow	Pale yellow-blue, pale bluish-purple, none	Pale yellow, pale orange-yellow, pale greenish-yellow
Live eggs	Greenish-yellow areas, none (dark against background)	Pale red, none	Yellow areas, orange-yellow areas, none (dark against background)	Pale bluish-purple	Pale yellow areas, pale greenish-yellow areas, none (dark against background)
Dead eggs	Green, orange-yellow, greenish-yellow	Red	Yellow-green	Blue, blue-green	Greenish-yellow
5. Not treated with stain					
Hatched eggs	Yellow	Pale red, none	Turquoise, yellow-green	Pale blue, pale blue-green	Yellow-green

Table 1. Continued.

Stain	Filter system*				
	B	G	V	UV	BV
Live eggs	Yellow	Red	Turquoise, yellow-green	Pale blue, pale blue-green	Yellow-green
Dead eggs	Yellow	Red	Turquoise, yellow-green	Blue, blue-green	Yellow-green, lime-green

* The filters and dichroic mirrors in each filter system are described in the text.

† Dead eggs were heat killed or had died of natural causes.

‡ In some eggs, no bright fluorescence was observed, but the eggs did stand out as dark objects against a bright background. Where the word "none" appears with no further description, the eggs were indistinguishable or barely distinguishable from the background.

fluorescence of the egg. These eggs sometimes resembled live, uninfected, dark eggs. With the BV filter system some hyphae fluoresced orange, but others were difficult to see.

Hyphae in fluorescein generally appeared non-fluorescent to dark. In most cases, it was difficult to determine with fluorescence microscopy whether hyphae were present. The UV system was an exception—some of the hyphae fluoresced faint blue.

In fluorescein diacetate, fluorescence was not observed in the fungal hyphae.

When infected eggs that had not been treated with any stain were observed, no fluorescence was seen in hyphae, except with the BV filter system. Some hyphae fluoresced faint yellow-green in the BV system.

Discussion

Of the stains used with bright-field optics, chrysoidin, eosin-Y, new blue R, and Nile blue A gave the clearest distinction between live eggs of *Heterodera glycines* and eggs that had been heat-killed or that had died of natural causes. The colors observed with chrysoidin were somewhat different from those previously reported by Ogiga and Estey (1974). These authors observed that dead nematodes and eggs stained yellow, whereas live specimens stained yellow with orange granules. In the current study, dead eggs were dark yellow (often with orange droplets) or orange, and live eggs were clear to yellow.

Acridine orange differentiated dead from live eggs with bright-field microscopy, but the distinction was not as easy to make as it was with the other stains. The major use of this stain for nematodes has been with fluorescence microscopy (e.g., Homeyer, 1953; Kurt, 1977; Perry and Feil, 1986).

With all of the stains some eggs could not be readily classified as either live or dead. This, along with the inability to stain certain types of dead eggs, has been mentioned before as a problem when nematodes were treated with some of these stains. For example, Moriarty (1964) observed that new blue R was good for nematodes killed by heat or chemicals, but not for nematodes less than 2 yr old that had died of natural causes in the soil. Sayre (1964) found that dead larvae that had been frozen and then warmed did not always stain well with new blue R. Ogiga and Estey (1974) found that new blue R results were not always consistent, while chrysoidin was a little more reliable, and Nile blue A was more reliable than either of these stains. These variable results indicate that the best stain to use may depend on several factors, including the source of the eggs and the means of egg death. It is interesting to note that in our experiments with bright-field microscopy, DMSO added to chrysoidin, eosin-Y, new blue R, and Nile blue A appeared to increase the contrast between live and dead eggs. When these 4 stains were mixed with DMSO and applied to eggs from inoculated cysts, similar percentages of live eggs were counted for each stain, indicating that these stains were equally reliable for distinguishing dead from live eggs. New blue R and Nile blue A might be advantageous in studies where it is necessary to clearly discern fungal hyphae, because these 2 stains caused the hyphae to stand out distinctly.

Forge and MacGuidwin (1986), examining plant-parasitic nematodes with the fluorescence microscope, found that live and dead nematodes could be distinguished from each other by patterns of autofluorescence. The major difference was that autofluorescence of live nematodes usually appeared as particles in the intestine, while

in dead nematodes, autofluorescence was diffuse throughout the body. Their observations were of nematodes that were older than the egg stage. In our study, live and dead eggs that had not been treated with a stain were mostly indistinguishable when examined with the fluorescence microscope. In the cases where the dead eggs tended to be brighter than the live eggs, the 2 egg types were still not easy to distinguish with a rapid scan, particularly when the eggs were in groups and both live and dead appeared fairly bright. However, the fluorescent stains acridine orange, fluorescein, and fluorescein diacetate were useful for distinguishing live from dead nematode eggs. Eosin-Y, which has not generally been used as a fluorescent stain for eggs of plant-parasitic nematodes, also differentiated between live and dead eggs with fluorescence optics. The colors or patterns of staining observed with fluorescence microscopy were not always similar to those reported by previous authors (Homeyer, 1953; Perry and Feil, 1986). This might be because different types of specimens were examined, or because different filter systems were used.

No single fluorescent stain was identified as consistently better than all of the others; a number of stain-filter system combinations distinguished live and dead eggs from each other. Excitation wavelengths that have been used by some authors studying nematode stains for fluorescence microscopy (Bird, 1979; Perry and Feil, 1986) and those provided by the manufacturer include blue and broad-band blue for acridine orange, green for eosin-Y, violet, blue, green, and ultraviolet for fluorescein compounds, and blue for fluorescein diacetate. A number of the filter systems that proved useful in this study were similar to these. One problem that needed to be taken into account was that stain-filter system combinations that distinguished between live and dead eggs were not always effective in differentiating dead eggs from hatched eggs. This was because some of the hatched eggs fluoresced, and the fluorescence colors were similar to those of dead eggs. Additional problems arose when fluorescence microscopy was used. Eggs that did not fluoresce could be overlooked when counting the total egg population. Hyphae in certain stain-filter system combinations did not appear to fluoresce, whereas in other combinations, the hyphae fluoresced the same colors as the infected eggs. Consequently, the fluorescent glow of an egg could obscure internal fungal hyphae. Uptake

of stains may vary with different fungi, but similar difficulties might have to be overcome with any fungus in such a study. If bright-field and fluorescence microscopy were combined to overcome these problems, increased time was necessary to count live and dead eggs. These limitations should be considered when selecting a fluorescent stain for eggs of plant-parasitic nematodes. Several filter system-stain combinations that have been shown to be useful may have to be tested to determine which one is the most reliable for the conditions of a particular experiment.

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